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(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Porous Microbead Permeable to Macromolecules Having Immobilized Therein at Least One Biological Particulate

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ABSTRACT OF THE DISCLOSURE

The present invention relates to a porous microbead having a size range between 100μ and 10mm and at least one biological particulate immobilized therein, said immobilized particulate being selected from the group consisting of microorganism, cell, a suspension of enzymes and a suspension of proteins, wherein said microbead is further characterized by having pores therethrough and wherein the average size of said pores ranges between 30Å and 1μ and allows the release of any macromolecule derived from said immobilized particulate to the outside of said microbead and the exchange of any other macromolecule between the outside and the inside of said microbead. There is also provided a preferred embodiment wherein said particulate is the microorganism P. pictorum which possesses the ability to breakdown cholesterol in a macromolecular cholesterol-lipoprotein complex, and wherein said cholesterol containing liquid is flowed through said microbead thereby causing the cholesterol of the cholesterol-lipoprotein complex which penetrate into said microbead from the outside medium of said microbead to be degraded by said immobilized microorganism or components thereof such as bacterial extracts and releasing said lipoprotein to the outside of said microbead.

TITLE OF THE INVENTION

A porous microbead permeable to macromolecules having immobilized therein at least one biological particulate.

BACKGROUND OF THE INVENTION

Microencapsulation of microorganisms or cells prior to their administration to the patient, prevents the direct contact between them and the patient's internal environment. Microorganisms are potentially health threatening and cannot be administered to a patient unless they are previously encapsulated. Cells can be rejected by the immune system of the body to which they are administered. Such microorganisms or cells may have an ability to produced, for instance, a desired protein or polypeptide lacking to a patient suffering from a given disease. Such encapsulated microorganisms or cells can also be used to metabolize and remove specific external molecules bound to macromolecules (e.g. cholesterol-lipoprotein complex). The encapsulation of this substance enables its administration to the patient in need and permits its removal if desired. A good microcapsule or microbead is one that has pores of a mean size such as to allow the exchange of macromolecules produced or degraded between the outside and the inside of said microbead.

Cholesterol is one of the most important factors causing the development of atherosclerosis. In fact, there is a strong correlation between the incidence of atherosclerosis and a high level of cholesterol

in blood. Cholesterol in blood is bound to macromolecules called lipoprotein. Furthermore, high concentrations of cholesterol in blood will eventually produce cholesterol deposits on the artery walls. These soft fatty deposits are atheromatous plaques which may be found in the main arteries of the legs, abdomen, heart and brain.

As atheromas grow, they may impede blood flow in affected arteries and damage the tissues they supply. Also, the plaque may provide a roughened surface, thereby leading to conditions which are suitable to include clot formation. An additional danger is that the plaque may rupture, resulting in thrombus and emboli formation. This could lead to the obstruction of small arteries and capillaries. Furthermore, atherosclerosis increases blood pressure by reducing the elasticity and narrowing the diameter of the arteries. Thus, resistance to the blood flow increases, thereby requiring greater work by the circulatory system to maintain the same level of blood circulation. The heart's work may increase, blood flow may decrease or a combination of the two may occur.

Cholesterol is present in all animal cells, blood and nervous tissue. It is a fundamental component of the cell membrane and it plays an important role in lipid transport. The metabolism of cholesterol is subdivided into anabolism or synthesis, and catabolism or degradation. When cholesterol enters the digestive apparatus, it combines with bile acids to produce a stable emulsion. It is then absorbed into the blood stream and transported by chilomicrons or lipoproteins. Cholesterol is a raw material for the production of

hormones, bile acids, D vitamin and cell membranes. It can be synthesized in the liver from other lipids or carbohydrates.

Accumulation of cholesterol in blood is usually caused by either excessive absorption through food, metabolic disorders such as hyperlipoproteinemia (HLP) type II, decrease in new cell production which accompanies aging or by a decrease in hormone production, especially for women.

Because of the importance of cholesterol in atherosclerosis, many researchers have been trying to find solutions to the problem of elevated cholesterol levels in blood. Reduction of cholesterol intake by a proper diet is probably the best method of atherosclerosis prevention. Such a diet may often be quite effective in lowering cholesterol levels in atherosclerotic patients. However, when the accumulation is greater as in the case of HLP type II, such a diet may not be sufficient.

In 1913, Sohngen was the first to report a microorganism growth using cholesterol as the only carbon and energy source (J. Gen. Microbiol., 1966, 45: 441-450). Later on, Haag reported another organism having this capability (J. Gen. Microbiol., 1966, 45: 441-450). However, the report of Sohngen and Haag were based on growth observations. In 1942, Tak (On Bacteria Decomposing Cholesterol, Antoine Van Leeuwenhoek, 1942, 8: 32-40) first proved that this growth was due to the utilization of cholesterol by measuring cholesterol concentrations in the final broth. This was the beginning of a new

research area focused on finding the biochemical pathway of cholesterol degradation.

Turfitt isolated and analyzed most of the intermediate products of the catabolism of the side chain of cholesterol. His findings are summarized in Biochem. J. 42: 376-383 (1948). He also reported the microbial fission of the nucleus. Sih et al. described most of the reactions to degrade the nucleus of the sterol (Ann. Rev. Biochem. , 1968, 37: 661-694). Thus, research has achieved a good understanding of the catabolic pathway of cholesterol, although the mechanism is not quite completely known. According to Talalay (Physiol. Rev., 1957, 37: 362-389), a chain of induced enzymes are responsible for the catabolism of cholesterol. The terminal pathway of oxidation of the intermediate products is the Krebs citric acid cycle. Cholesterol side chain in nucleus degradation may be simultaneous unless an inhibitor is present. Gray and Thornton (Soil Bacteria that Decomposes certain Aeromatic Compounds Zentbl. Bakt. Parasitkde, 1928, (Abt 2), 73:74-96) found that Pseudomonas pictorum was capable of degrading phenol. The National Collection of Industrial Bacteria Catalogue of Strains states that P. pictorum degrades cholesterol.

Thus, it appears clear that cholesterol degradation to carbon dioxide and water under microbial attack is possible. Voet et al. used this fact to decrease blood cholesterol level of dogs 'in vitro'. They used a resting suspension of Pseudomas species in dog blood plus 0.1% cholesterol. All cholesterol was metabolized in 3 days and no intermediate products were detected. However, it is likely that antibody reactions would appear if microorganisms or free cell extracts

were used directly in animal or human. Immobilization of these microorganisms inside microbeads would prevent their direct exposure to animal or human. However, microbeads which are permeable to macromolecules such as cholesterol-lipoprotein complex are required for these microorganisms to be able to operate while being encapsulated.

Rao B.S. et al. describe a method of immobilization which is exclusively related to the entrapment of yeast, which is a rather large cell (Applied Biochem. and Biotech., 1986, 12:17-24). There is no teaching in this article of the effective immobilization of smaller cells, such as bacteria, which would be without any leakage. The open pore agar matrix prepared by Rao B.S. et al. is not optimized with respect to the temperature and to the alginate concentration required for its production. There is not even mentioned the autoclaving time required as well as the effective diffusability of macromolecules such as cholesterol-lipoprotein complex. Further, this entrapment method has not been tested for the mass transfer of small molecules such as glucose or large molecules such as protein or lipoprotein. There is only observed in this article, the rupture of these alginate beads due to the evacuation of the carbon dioxide produced by the immobilized yeast.

The ideal immobilization method depends very much on the application. However, certain properties are desirable.

First, the physical confinement of the cells is important. The carrier should not only effectively retain the entrapped particulate but should also accept high cell loadings (10^9 cells/mL or more). It

should be chemically and physically stable as well as shear resistant The diffusion of nutrients to the entrapped cells play a key role in the global reaction rate. Thus, the microbead should minimize diffusion resistances and be permeable to macromolecules such as the cholesterol-lipoprotein complex.

Second, the method must preserve the catalytic activity and, if possible, the viability of the entrapped particulate (e.g. microorganism). The procedure should be mild and physiologically compatible with the particulate to be entrapped. Thus, it is important to avoid conditions such as high temperatures, extreme pH, toxic chemicals or high shear rates.

Therefore, it would be would be highly desirable to have a microbead which would permit the high diffusion of macromolecules between its inside medium and its outside medium and which would even permit the entry of cholesterol-lipoprotein complex.

Furthermore, it would be would be highly desirable to have a microbead which would permit complete degradation of excess cholesterol in blood without undesirable side effects.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a porous microbead which is obtained from the optimization of the temperature as well as the pore-forming material (e.g. alginate) concentration required for its production and which would overcome the above-mentioned drawbacks.

In accordance with the present invention, there is provided a porous microbead having a size range between 100μ and 10mm and at least one biological particulate immobilized therein, the immobilized particulate being selected from the group consisting of microorganism, cell, a suspension of enzymes and a suspension of proteins, wherein the microbeadis further characterized by having pores therethrough and wherein the average size of the pores ranges between 30Å and 1μ allows the release of any macromolecule derived from the immobilized particulate to the outside of the microbead and the exchange of any other macromolecule between the outside and the inside of the microbead.

In a preferred embodiment of the present invention, there is provided a microbead wherein the immobilized particulate is the microorganism P. pictorum which possesses the ability to breakdown cholesterol in a macromolecular cholesterol-lipoprotein complex, and wherein the cholesterol containing liquid is flowed through the microbead, thereby causing the cholesterol of the cholesterol-lipoprotein complex which penetrates into the microbead from the outside medium of the microbead, to be degraded by the immobilized

microorganism or components thereof such as bacterial extracts and releasing the lipoprotein to the outside of the microbead.

In another preferred embodiment of the present invention, the macromolecule derived from the particulate is selected from the group consisting of protein, antibody, antigen, polysaccharide, lipoprotein and polypeptide.

In another preferred embodiment of the present invention, the microbead is made of a material selected from the group consisting of agar and agarose in association with a pore-forming agent.

There is also provided, a method of producing the microbead of the present invention, which comprises the steps of:

a) mixing a material selected from the group consisting of agar, carrageenan and agarose with a pore-forming agent;

b) autoclaving said mixture at 121°C for 15 minutes to increase the permeability during the next step;

c) cooling the autoclaved mixture to the desired temperature prior to entrapping at least one particulates in a microbead by using said mixture; and

d) causing the pore-forming agent to leach out from the mixture by using the appropriate solvent to form pores through the microbead.

The advantages of microencapsulation of the microorganisms and cells are numerous. First, microencapsulation of

the microbial cells or cells separates the bacteria or cells from blood or body fluid cells by preventing a direct contact between blood or body fluid cells and the bacteria or cells. Furthermore, removal of the microencapsulated bacteria after use can be easily accomplished. This same approach can also be used for the removal of cholesterol from milk or other biological fluids.

The particulars of the present invention are detailed in the following description.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a porous microbead having a size range between 100μ and 10mm and at least one biological particulate immobilized therein, the immobilized particulate being selected from the group consisting of microorganism, cell, a suspension of enzymes and a suspension of proteins, wherein the microbead is further characterized by having pores therethrough and wherein the average size of said pores ranges between 30Å and 1μ allows the release of any macromolecule derived from the immobilized particulate to the outside of said microbead and the exchange of any other macromolecule between the outside and the inside of the microbead.

In a preferred embodiment of the present invention, there is provided a microbead wherein the immobilized particulate is the microorganism P. pictorum which possesses the ability to breakdown cholesterol, and wherein the cholesterol containing liquid is flowed through the microbead, thereby causing the cholesterol of the cholesterol-lipoprotein complex which penetrates into the microbead

from the outside medium of the microbead, to be degraded by the immobilized microorganism or components thereof such as bacterial extracts and releasing the lipoprotein to the outside of the microbead. These cholesterol molecules may or may not be bound to carriers such as lipoproteins.

Particulate

In accordance with the present invention, the biologically active particulates which can be immobilized within the microbead are selected from the group consisting of microorganism, cell, a suspension of enzymes and a suspension of proteins.

Cells

Concerning the immobilization of cells, a microbead which is produced under a lower temperature is required for the cells to remain alive.

Among the cells that may be used in accordance with the present invention, there may be mentioned liver cells, islet cells, endocrine cells, hybridomas, virus and yeast.

Proteins

Among the proteins that may be secreted by cells immobilzed in the microbeads of the present invention, there may be mentioned antibodies, antigens, peptides, clotting factors and growth factors.

Enzymes

Among the suspensions of enzymes that may be used in accordance with the present invention, there may be mentioned

suspension from extracts and intracellular organelles prepared from cells or microorganisms.

Whole cells versus enzymes

Immobilized cells have many advantages over immobilized enzymes. The main advantage is the elimination of the enzyme purification step. Furthermore, many enzyme reactions require cofactors that are very complicated and expensive to purify. Viable whole cells can produce these cofactors and regenerate them. Enzymes are also more stable in the intracellular environment than in their purified form. This includes greater resistance to physical changes such as temperature and/or pH and chemical changes such as the oxydizing substances or metals. On the other hand, immobilized enzymes have some advantages. A given enzyme carries out a specific reaction and usually no side reaction occurs. Microbes have complex biochemical pathways, and side reactions are possible. Cells require nutrients such as oxygen to stay alive. Cells can synthesize and secrete desirable products such as proteins, antibodies, peptides and growth factors.

Microorganisms

Various microorganisms possess desirable properties which may be useful as medical treatments, one of these desirable properties being the ability to deplete cholesterol. Among the microorganisms that may be used in the context of the present invention for the purpose of depleting free cholesterol or cholesterol bound to lipoproteins, there may be mentioned P. pictorum, Lactobacilus acidophilus, Nocardia erythropolis and Pseudomonas

species among others and mixtures thereof. These microorganisms are all readily available from the American Type Culture Collection. The microorganism can be cultured in a suitable culture medium such as bovine or calf serum. The bacteria are harvested by standard harvesting methods such as centrifugation or filtration, washed with a suitable saline solution and resuspended in water. The microorganism concentration is measured in terms of protein contained in the bacteria. Protein levels are measured by techniques such as the Biuret Method. Although the proportion of proteins contained in the assayed bacteria is not known, the maintenance of similar total solution of unknown protein concentrations permits the repeat use of identical bacteria concentrations. Once the desired amount of microorganism is obtained, which corresponds to a protein concentration varying between 2 and 4g/dL, they are then ready to be encapsulated in porous microbeads. Once the bacteria have been encapsulated, the microbeads can be contacted with the material to be depleted of cholesterol by numerous contacting methods known by those skilled in the art such as contact in a reactor, or by ingestion by a patient having a high cholesterol count.

Microbeads

The microbeads that may be employed in the context of the present invention are matrices possessing pores that are large enough to permit the entrance of macromolecules such as cholesterol-lipoprotein complexes. Thus, the pore size of the microbeads may vary between 30Å and 1μ. Therefore, a molecule like the cholesterol-lipoprotein complex will be able to diffuse in the microbeads and the

immobilized bacteria will degrade the cholesterol molecule allowing the free lipoprotein as well as the byproducts resulting from the cholesterol catabolism to diffuse out of the microbeads.

Thermal gelation

There are some natural polymer aqueous solutions that gel when the temperature is lowered. The gelling temperature of most ofd these polymers is lower than the melting temperature and varies from 15 to 45°C. All these properties make them ideal candidates for cell entrapment.

Among the natural polymers which may be used to form the microbeads there may be mentioned agar, agarose, carrageenan and other suitable polymers.

The general method for preparing microbeads consists in the emulsification of an aqueous polymer solution with a hydrophobic phase such as paraffin oil followed by a decrease in temperature of the emulsion below the gelling temperature and finally the resulting beads are washed.

Agar and agarose are resistant to microbial attack and form porous gels. Agar solidifies at a temperature of about 42°C, while agarose solidifies between 15°C and 40°C.

An agar solution having a 1 to 4% agar concentration is the preferred matrix-forming agent for the immobilization of microorganism, suspension of enzyme and suspension of proteins.

An agarose solution having a 2 to 4% agarose concentration is the preferred matrix-forming agent for the immobilization of cells.

Ionotropic gelation

Pore-forming agent are used for extremely mild ionic cross-linking reaction to form gels out of natural polymers. Porous microbeads will be obtained by adding a pore-forming agent that will be admixed within the polymer microbead.

Pore-forming agents such as sodium alginate, gelatin and others may be used in the context of the present invention.

Once the matrix has been crystallized, the pore-forming agent is leached away by a suitable leaching agent such as 1 to 5% sodium citrate solution, thereby leaving a network of pores throughout the matrix.

It will be readily understood that the pore size will essentially depend on the concentration of the pore-forming agent as well as the temperature under which the reaction is carried out. A small concentration of pore-forming agent will lead to the formation of small pores while larger concentrations will result in larger pores.

Preferably, sodium alginate is employed as the pore-forming agent. The total concentration of sodium alginate in the mixture will usually range from 2 to 5%, being preferably 2%.

Immobilization of microorganism

Suitable microorganisms may be entrapped in the microbeads in the following general manner: 5 to 30mL of a solution containing the bead forming material, which are preferably composed of 2 to 4% of agar and 2 to 5% of sodium alginate, are slowly added 0.5 to 5mL of an aqueous suspension containing the microorganism to be entrapped. The concentration of microorganism of this solution, when measured as a protein concentration, usually ranges between 2 and 4g/dL.

It is noted that continuous mixing is required in order to avoid crystallization of the material forming the beads. Once the solution containing the microorganism suspension and the material used to form the beads has been prepared, it is extruded, for example through a previously sterilized syringe pump, into a solution containing 50 to 300mL of a crystallizing agent such as a 1 to 5% calcium chloride solution. Reagents that may be used to previously sterilize the syringe pump include Roccal® (400 ppm) as well as any suitable disinfectant.

The resulting beads are separated by conventional separation techniques, such as decantation and filtration, washed with a saline solution and stored in a suitable solution such as phosphate buffer saline. It will be readily understood that the quality of the performance of the microorganism-containing beads will depend mainly on the type of microorganism entrapped within the matrices.

Cholesterol depletion may then be effected by reacting a cholesterol containing liquid such as milk, blood, plasma and the like

in the presence of microbeads containing microorganism, like *P. pictorum*, having the ability to use cholesterol as a carbon source. The reaction can be monitored by any suitable method such as the analysis of cholesterol and intermediate products. Reaction time required to degrade approximately 75% of the cholesterol contained in a given solution ranges between 60 and 80 hours for an immobilized microorganism. However, a considerable decrease in that reaction time could be obtained by allowing the beads to stand in a concentrated cholesterol containing solution prior to reaction with the targetted liquid, thereby avoiding the lengthy initiation period required for the microorganism to start degrading cholesterol at reasonable rates. Furthermore, there is no limit to the amount of cholesterol that can be degraded by the method of the present invention. In fact, cholesterol is degraded faster in liquid possessing high cholesterol concentrations than in liquid possessing low initial cholesterol concentrations.

The process of the present invention will be more readily illustrated by referring to the following examples which do not intend to limit the present invention thereto.

Example I

Preparation of porous agar-alginate microbeads

A solution of 2% agar (sold by Difco) and of 2% sodium alginate (sold by Kelco) is autoclaved for 15 minutes at 121°C. The autoclaving time is important for the obtention of porous microbeads having large pore size. The solution is cooled to 45°C. *P. pictorum* is suspended in 0.4mL of 0.9% NaCl solution. This *P. pictorum*

suspension is added dropwise to 3.6mL of the agar-alginate solution at 45°C, while stirring vigorously. Then, 3mL of the resulting mixture are extruded through a previously sterilized syringe pump, into a solution containing 50 to 300mL of a crystallizing solution of 2% calcium chloride. Reagent that is used to previously sterilize the syringe pump is Roccal® desinfectant (400 ppm). It is important to keep the temperature at 45°C to avoid the gelation of agar in the conduits. The drops are collected in cold (4°C) 2% calcium chloride solution and allowed to harden. After 15 minutes, the supernatant is discarded and the beads are suspended in a 2% sodium citrate solution for 15 minutes. They are then washed and stored in a 0.9% saline solution at 4°C.

Leakage test of these microbeads

It is critical that immobilized particulate such as microorganism do not leak out of the microbeads. Studies carried out showed no leakage from these microbeads. The experimental parameters of each group of the study are shown in Table I.

Table I

Leakage Test : Experimental Conditions

Group Number	Microbead Agar mixture	Mcroorg. Suspension	Citrate Sln.	Saline Sln.
10	0.0	0.1	0.0	5.0
11A	0.9	0.1	0.0	5.0
11B	0.9	0.1	0.0	5.0
12A	1.0	0.0	0.0	5.0
12B	1.0	0.0	0.0	5.0
13A	0.9	0.1	5.0	0.0
13B	0.9	0.1	5.0	0.0
14A	1.0	0.0	5.0	0.0
14B	1.0	0.0	5.0	0.0

The results are summarized in Table II.

Table II

Leakage Test : Results

Group Number	Microorganism	Wavelength	Absorbance
10	free	400	0.1
		500	0.08
		600	0.06
		700	0.045
11	immobilized	400	0.003
		500	0.002
		600	0.002
		700	0.002
12	immobilized	400	0.003
		500	0.002
		600	0.002
		700	0.002
13	immobilized	400	0.003
		500	0.002
		600	0.002
		700	0.002
14	immobilized	400	0.002
		500	0.002
		600	0.002
		700	0.002

There can be observed from these results that the micobeads of the present invention show substantially no leakage of the immobilized microorganism.

Example II

Preparation of porous agarose-alginate microbeads containing mammalian cells

The microbead which is produced as described in Example I requires a higher temperature which is not optimal for most

mammalian cells. The following approach is based on agarose-alginate which requires a lower melting temperature which is more suitable for mammalian cells.

Among mammalian cells which can be encapsulated according to this procedure, there may be mentionned erythrocytes, leucocytes, platelets, hepatocytes, islet cells, hybridomas and others.

4g of agarose (A-3038® sold by Sigma) and 2g of sodium alginate (Leltone LV® sold by Kelco) are dissolved in 100mL of saline solution (0.9g of NaCl/100mL of water). The resulting solution is stirred at 80°C for about 3 hours. The solution is cooled by placing it in an ice bath while stirred until it reaches the temperature of 37°C. At this time 2 volume of this solution is mixed with 0.5 mL of a mammalian blood containing cell suspension (e.g. erythrocyte, leucocytes and platelets). The resulting suspension is added dropwise to a cool (4°C) calcium chloride solution (1.11g CaCl/100mL water). The microbeads are allowed to harden at 4°C for 30 minutes. Then the microbeads are washed three times with a saline solution at 4°C. They are then placed in a sodium citrate solution (1.47g Na citrate/100mL water) for one hour and this is repeated twice. The beads having immobilized therein the mammalian cells are then washed with saline and resuspended in saline.

The permeability of the resulting beads depend on the concentration of alginate used and the proportion which is leached out. This microbead can be produced at the physiological temperature of 37°C. After its preparation, the microbeads can be stored at even lower

temperature. These microbeads are permeable to proteins including hemoglobin which has a molecular weight of 68,000. A qualitative test can easily be perform wherein empty microbeads or microbeads having therein cells immobilized are suspended in a saline solution containing hemoglobin. Because hemoglobin is red its transfer can easily be visualized. After a while, the microbeads were removed from the solution and washed with a saline solution. The resulting microbeads were red in color thus showing their permeability to hemoglobin.

Example III

Preparation of porous microbeads containing P. pictorum

P. pictorum (ATCC 23328) were cultured first in Difco nutrient broth at 25°C, followed by harvesting and resuspension in a cholesterol medium. The composition of the cholesterol medium used is listed in Table III.

Table III

Cholesterol medium

Amonium nitrate	0.1%
Potassium phosphate	0.025%
Magnesium sulfate	0.025%
Ferric sulfate	0.0001%
Yeast extract	0.5%
Cholesterol	0.1%

After culturing this suspension for 15 days at 25°C, it was used as an inoculum for biomass production. The culture was grown in 50mL of bovine calf serum (sold by Sigma) at 37°C for 36 hours. After harvesting and washing the cells in 50mL of sterile water, the

cells were resuspended in water to obtain a concentration of bacteria measured as a protein concentration of 2.8g/dL. A standard extrusion apparatus was sterilized with 100mL of desinfectant solution (Roccal®) and washed with 100mL of sterile water to remove traces of the desinfectant. It was then heated at 45°C using a heating coil and a rheostat.

A solution of 2% agar and 2% sodium alginate was prepared, autoclaved for 15 minutes at 121°C and cooled to 50°C. Under vigorous stirring, 1mL of the bacteria suspension previously described was added dropwise to 10mL of the agar-alginate solution. It is to be noted that the stirring of the solution is extremely important to avoid the crystallization of the agar present in the solution. A 10mL syringe was then filled with this solution. The syringe was then immediately placed in a Harvard® syringe pump and readily surrounded by the heating coil. The syringe pump was then set at 2mL/min and started. The first 5 to 10 drops were discarded and the rest of the solution was collected in a sterile beaker containing 100mL of a 2% solution of cold calcium chloride while gently stirred with a magnetic bar. Once the solution was completely extruded, the beads were allowed to stay in the calcium chloride solution for another 15 minutes under gentle stirring. They were then separated by decantation and resuspended in 100mL of 2% of sodium citrate for one hour to leach out the alginate. Finally, the beads were washed with 100mL of phosphate buffer saline (PBS) and stored in PBS.

Example IV

Cholesterol depletion using porous microbeads containing P. pictorum

Immobilized P. pictorum is able to deplete serum cholesterol. Cholesterol depletion properties of the embedded bacteria were assessed by performing four separate experiments in which 1.8mL of beads containing bacteria were reacted with 10mL of bovine calf serum placed in a 50mL sterile erlenmayer provided with a foam plug cap to allow diffusion of the gases. 166 μL of the bacterial solution described in Example III were added to each sterile erlenmayers containing 10mL of bovine calf serum. For both free and entrapped bacteria solution, the initial amount of proteins representing the bacteria concentration was calculated to be 4 X 10^{-4}g. Between 1.4 and 1.8mL of empty beads in 10mL of bovine calf serum containing approximately 10mg of cholesterol were placed in a 50mL sterile erlenmayer with a foam plug cap and were used as controls. The flasks were incubated in a Lab-Line® orbital shaker at 37°C. Samples were collected every 12 hours and frozen for a later analysis. Analysis of the remaining cholesterol levels may be performed by using the Multistat III® Plus centrifugal analyzer. The reaction used is an enzymatic colorimetric reaction which is highly specific for cholesterol. Thus, 0.150mL of cholesterol specific enzyme reagent is added to 2 μL of each collected sample with 38μL of water. The analyzer provides temperature and mixing controls, and performs absorbance readings at specified reaction times and wavelength. The difference in absorbance between 500 and 690nm is proportional to the cholesterol concentration within the linear range. The remaining protein was

analyzed using the Biuret method which is a well known colorimetric chemical method. The Multistat III® Plus centrifugal analyzer was also used to perform the analysis.

It was observed that the initial decrease in cholesterol concentrations was more pronounced when the bacteria were immobilized in the agar matrix. This rapid initial decrease in concentration is probably due to the absorption of cholesterol within the agar matrix since a slower decrease is observed after the first ten hours, suggesting that the maximum absorption capacity has been achieved. It was also noted that the final cholesterol concentration was significantly higher when free bacteria were used than immobilized bacteria. The solution containing bacteria in a free form were centrifuged and then analyzed, while the solution containing the beads were analyzed after the removal of the beads. Thus, these results suggest that bacteria stores cholesterol, and if it is not separated from the media, a higher cholesterol concentration will be measured.

Results are summarized in Tables IV, V and VI.

TABLE IV

CHOLESTEROL DEPLETION TIMES FOR FREE *P. PICTORUM*

Experiment	Amount of bacteria added(ul)	Amount of protein contained in bacteria solution (g)	Time hours	% of initial cholesterol remaining
1	166	4.4×10^{-3}	0	100.0
			10	78.0
			20	78.9
			30	77.1
			40	70.2
			50	66.1
			60	67.3
2	166	4.4×10^{-3}	0	100.0
			12	88.5
			24	69.2
			36	46.8
			48	37.8
			60	34.0
3	166	4.4×10^{-3}	0	100.0
			12	98.3
			24	96.6
			36	93.2
			48	80.3
			60	57.0
4	166	4.4×10^{-3}	0	100.0
			12	93.8
			24	80.9
			36	56.1
			48	45.2
			60	41.6

Note: the concentration of bacteria in solution is proportional to the amount of protein measured.

TABLE V

CHOLESTEROL DEPLETION TIMES FOR ENCAPSULATED P. PICTORUM

Experiment	Amount of bacteria added(ul)	Amount of protein in bacteria solution (g)	Time hours	% of initial cholesterol remaining
1	1.8	4.4×10^{-3}	0	100.0
			10	73.7
			20	66.3
			30	66.3
			40	48.8
			50	45.0
			60	50.9
2	1.8	4.4×10^{-3}	0	100.0
			12	64.9
			24	43.1
			36	22.8
			48	16.8
			60	16.2
3	1.8	4.4×10^{-3}	0	100.0
			12	76.4
			24	66.6
			36	60.8
			48	50.4
			60	24.6
4	1.8	4.4×10^{-3}	0	100.0
			12	57.7
			24	37.9
			36	21.4
			48	12.5
			60	12.2

Note: the concentration of bacteria in solution is proportional to the amount of protein measured.

TABLE VI

LEVELS OF CHOLESTEROL ACCUMULATED IN FREE P. PICTORUM

Time hours	% chol. remaining in solut. after centrifug.	Serum % chol. remain. prior to centrifug.	Serum protein	Bacteria % chol. found in	Bacteria protein
0.0	100.0	100.0	5.45	0.0	0.0
22.0	90.0	89.0	5.40	4.7	0.16
68.0	64.5	39.2	5.87	29.5	1.37
73.5	59.0	32.7	6.10	32.0	1.70
90.5	45.0	12.0	5.75	36.8	2.12

WHAT IS CLAIMED IS:

1. A porous microbead having a size range between 100μ and 10mm and at least one biological particulate immobilized therein, said immobilized particulate being selected from the group consisting of microorganism, cell, a suspension of enzymes and a suspension of proteins, wherein said microstructure is further characterized by having pores therethrough and wherein the average size of said pores ranges between 30Å and 1μ and allows the release of any macromolecule derived from said immobilized particulate to the outside of said microbead and the exchange of any other macromolecule between the outside and the inside of said microbead.

2. The microbead of Claim 1, wherein said wherein said macromolecule derived from said particulate is selected from the group consisting of protein, antibody, antigen, polysaccharide, lipoprotein and polypeptide.

3. The microbead of Claim 1, wherein said microbead is made of a material selected from the group consisting of agar, carrageenan and agarose in association with a pore-forming agent.

4. The microbead of Claim 1, wherein said particulate is the microorganism P. pictorum which possesses the ability to breakdown cholesterol in a macromolecular cholesterol-lipoprotein complex, and wherein said cholesterol containing liquid is flowed through said microbead thereby causing the cholesterol of the cholesterol-lipoprotein complex which penetrate into said microbead from the outside medium of said microbead to be degraded by said

immobilized microorganism or components thereof such as bacterial extracts and releasing said lipoprotein to the outside of said microbead.

5. The microbead of Claim 1, wherein said particulate is a cell which is capable of secreting macromolecules selected from the group consisting of proteins and antibodies to the outside of said microbead.

6. A method of producing the microbead of Claim 1, which comprises the steps of:

a) mixing a material selected from the group consisting of agar, carrageenan and agarose with a pore-forming agent;

b) autoclaving said mixture at 121°C for 15 minutes to increase the permeability during the next step;

c) cooling the autoclaved mixture to the desired temperature prior to entrapping at least one particulates in a microbead by using said mixture; and

d) causing the pore-forming agent to leach out from the mixture by using the appropriate solvent to form pores through the microbead.